Critical occurrence of verotoxigenic E. coli and non-typhoidal salmonella in some heat-treated dairy products

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Abstract
Pathogenic strains of E. coli and Salmonella are common causes of foodborne illness and have been frequently isolated from inadequately heat-treated milk products in Mansoura city. The current study was performed to explore the prevalence of E. coli and Salmonella spp. in heat-treated milk products intended for consumption in Mansoura university hospitals and hostels, as well as, to investigate their serotypes and virulence potential. Seventy-five samples of heat-treated milk products (Soft cheese, yoghurt, and processed cheese, 25 of each) were randomly gathered with the benefit of prolonged shelf life requirements either for the development and intense growth of children or for lessening the occurrence of chronic illnesses (osteoporosis, type II diabetes, hypertension, and cancer) in adolescents (Salles et al., 2019). Lately, cheese constituted the main part of patient meals being an energy-rich nutritious, and a concentrated form of milk with the benefit of prolonged shelf life (El-Zamkan et al., 2019). Moreover, yoghurt is a staple food in several cultures being an excellent source of probiotic microorganisms (viable Lactobacillus acidophilus) and lysozyme which can improve the immune response, antitumor effect, and encourage better assimilation of nutrients (Loureis-Hattingh and Viljoen, 2001). Despite this, milk products may harbor hazardous microbes such as E. coli and Salmonella, if they were improperly heat-treated and unsanitary processed which make them unfit or even threatening sources of foodborne illness (Singhal et al., 2020).
In fact, pathogenic strains of E. coli and Salmonella were identified frequently to be the common foodborne pathogens linked to consumption of raw or inadequately heat-treated milk products sold in rural areas and local groceries of Mansoura city (El-Baz et al., 2017; Omar et al., 2018; Elafify et al., 2020). The most prevalent E. coli serotypes related to human foodborne illnesses are; enteropathogenic E. coli (EPEC), enterotoxigenic E. coli (ETEC) as well as enterohemorrhagic E. coli (EHEC) which is known as lethal shiga toxin or verotoxin producing E. coli (STEC or VTEC) (Hussien et al., 2019). STEC is a zoonotic diarrheagenic pathotype of E. coli causing approximately 2,801,900 moderate to severe gastrointestinal disorders, hemolytic uremic syndrome (HUS), and hemorrhagic colitis (HC) each year giving rise to serious public health burden worldwide (Galace et al., 2019). The distinguishing characteristic of STEC is the presence of one or two major forms of Shiga-like toxins encoding genes such as stx1 and stx2 (verotoxin) genes together with enterohemolysin (hly) and intimin (eaeA) virulent genes that intensify its pathogenicity in causing infections (El-Baz et al., 2017). Although the majority of sporadic cases and outbreaks of STEC/EHEC are still attributed to O157:H7 serotype, nowadays the big six serotypes (O45, O26, O103, O121, O111, and O145) of non-O157 STEC showed a marked increase in incidence rates in Egypt (Elmonir et al., 2021) and worldwide (Gould et al., 2013; Loconsole et al., 2020).

Introduction
Milk products are rich sources of necessary nutrients that achieve nutritional daily requirements either for the development and intense growth of children or for lessening the occurrence of chronic illnesses (osteoporosis, type II diabetes, hypertension, and cancer) in adolescents (Salles et al., 2019). Lately, cheese constituted the main part of patient meals being an energy-rich nutritious, and a concentrated form of milk with the benefit of prolonged shelf life (El-Zamkan et al., 2019). Moreover, yoghurt is a staple food in several cultures being an excellent source of probiotic microorganisms (viable Lactobacillus acidophilus) and lysozyme which can improve the immune response, antitumor effect, and encourage better assimilation of nutrients (Loureis-Hattingh and Viljoen, 2001). Despite this, milk products may harbor hazardous microbes such as E. coli and Salmonella, if they were improperly heat-treated and unsanitary processed which make them unfit or even threatening sources of foodborne illness (Singhal et al., 2020).

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Materials and methods

Samples collection

A total number of seventy-five samples from different batches of dairy foods (soft cheese, yoghurt, and processed cheese, 25 of each), were randomly collected in clean, dry, and sterile containers, from the food department of Mansoura hospitals and hostels, and this by investigating the presence of E. coli and Salmonella strains in randomly collected milk product samples with attention to the characterization of their serotypes and virulence potential.

Identification and characterization of the isolated E.coli and Salmonella strains

Biochemical identification

After refreshment of preserved isolates, biochemical identification was performed according to Hendriksen (2011) using triple sugar iron, indol production, hydrogen sulphide, citrate utilization, voges-proskauer, urease, and methyl red tests.

Serotyping

Biochemically confirmed E.coli and Salmonella strains were serologically typed into serovars by slide agglutination method using rapid diagnostic polyvalent and monovalent E.coli and Salmonella agglutinating antisera sets (Denka Seiken®, Tokyo, Japan) as per Forbes et al. (2007).

Molecular characterization

Bacterial genomic DNA extraction and purification were performed using QIAamp DNA Mini Kits (Cat#51304, Qiagen®, GmbH, Hilden, Germany) as per the manufacturer’s protocol. In brief, each isolate suspended in enrichment broth (200 μl) was lysed for 2 h at 56°C with Qiagen® protease (20 μl) then lyses process was stopped by adding AL buffer (200μl) for 10 min at 56°C. After centrifugation, 200 μl of ethanol (96%) was added to the DNA containing supernatant and the obtained mixture was passed through the QIAamp kit column. The purity of DNA bounded to a QIAamp membrane was improved after two washes using AW1 and AW2 (wash buffers). The purified DNA was then eluted in AE (50 μL) elution buffer and stored at -80°C until investigated for the presence of E. coli and Salmonella virulence genes.

PCR was applied via a thermocycler (Applied Biosystems Geneamp 2720) using 6 μl of the eluted DNA, 12.5 μl of Emerald Amp GT PCR master mix (2X premix,
TAKARA Bio Inc. Cat# RR310A), 1 μl of each forward and reverse primer as well as 4.5 μl of PCR grade water. The primer pairs (Table 1) that were used for the identification of virulence genes were designated by Metabion (Martinsried, Germany) whereas; positive control strains were obtained from Animal Health Research Institute, Dokki, Egypt.

The amplification reaction was applied as per the following protocol: primary denaturation at 94 °C for 5 min; followed by 35 cycles of secondary denaturation at 94 °C for 30 sec; annealing at 58 °C for 40 sec for stx1, stx2 and spvC at 51 °C for 30 sec for eaeA, at 60 °C for 40 sec for hly, at 59 °C for 40 sec for stn and 55 °C for 30 sec for invA gene; extension at 72 °C for 45 s in stx1, stx2, stn, and spvC, at 72 °C for 30 s in eaeA and invA, at 72 °C for 60 s in hly. Finally, the PCR amplified products were visualized under UV light and photographed after electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) stained with ethidium bromide.

### Results and discussion

The lack of sanitary measures and individual hygiene during the manufacturing of some dairy products makes them the primary reservoir of *E. coli* that represents an insidious threat to human health and food safety (Mortaz et al., 2012). The result of our investigation (Table 2) revealed that 3/25 (12%) of examined soft cheese samples were positive for *E. coli* and this incidence nearly agrees with Stephan et al. (2008), and Elbaz et al. (2019) who revealed that 5/52 (9.6%) and 3/30 (10%) of examined samples in Switzerland and Egypt, respectively, were positive for *E. coli*. Another study from Brazil stated the high prevalence of *E. coli* (95.5%) especially O26:H11 serotype in different soft cheese brands. In our study, *E. coli* isolates were serologically identified as O146:H21, O26:H11 and O128:H2 serovars that similar to serotypes previously detected in soft cheese during another survey in the same city by Elhadidy and Mohammed (2013) who isolated O146:H21 and O26:H11 whereas, O128:H2 was previously isolated from soft cheese in Iran (Mortaz et al., 2012). In the same way, our study showed that 2/25 (8%) of examined yoghurt samples were tainted with *E. coli* O128:H2 and O121:H7 serotypes that also identified in other study conducted in Iran by Dekhordi et al. (2014).

Hence, based on our survey and the most recent result of *E. coli* serotypes prevalence in yoghurt (Elbaz et al., 2019) and soft cheese (Elafify et al., 2020; Elbaz et al., 2019) sold at the retail level in Mansoura city, we noted that it is the second time to isolate O146 and O26 from soft cheese since seven years ago by Elhadidy and Mohammed (2013) and it is the first time to isolate O128 and O121 from yoghurt in the same city.

In an attempt to assess the pathogenicity of these serotypes, virulence factors were further determined by PCR and our findings (Table 2, Figure 1) confirmed the presence of *eaeA* virulence gene in all isolated serotypes that have a role in enhancing immediate adherence of microorganism to the intestinal wall resulting in the attaching and effacing (A/E) lesions after disruption of intestinal microvillus brush border (Blanco et al., 2005). Despite this, our result stated the low pathogenicity of EPEC (O128 and O146) due to the absence of *stx*2, *stx*1 and *hly* virulence genes but also, our results confirmed the emergence of highly pathogenic non-O157 VTEC in soft cheese (O26) and yoghurt (O121) as they harbored and expressed *stx*2 (Verotoxin) virulence gene that usually implicated as a cause of sporadic cases or outbreaks of bloody diarrhea, HUS and renal insufficiency as it thousand times more toxic for renal microvascular endothelial cells than *stx*1 especially in the presence of *eaeA* gene that has an accessory role in augmenting the pathogenicity of STEC (EFSA, 2013).

Henceforth, virulence gene profile suggested that STEC positive products were manufactured from improperly pasteurized milk that previously polluted either from the farm environment or contaminant in the dairy chain as Cattle are a major reservoir of highly virulent STEC strains that carry *stx*2 and *eaeA* with high rates than *stx*1 and *hly* (Karama et al., 2019).

Non-typhoidal *Salmonella* is a major microbiological hazard associated with the consumption of dairy products made either

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**Table 2. Prevalence, Serodiagnosis, and Virulence genes of *E. coli* isolated from soft cheese and yoghurt that consumed in Mansoura university hostels and hospitals.**

<table>
<thead>
<tr>
<th>Type of samples (No.)</th>
<th>Prevalence No. (%)</th>
<th>Pathotype</th>
<th>Serodiagnosis</th>
<th>Virulence genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>stx1</td>
<td>stx2</td>
</tr>
<tr>
<td>Soft cheese (25)</td>
<td>3 (12)</td>
<td>EPEC</td>
<td>O146:H21</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>STEC</td>
<td>O26:H11</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EPEC</td>
<td>O128:H2</td>
<td>-</td>
</tr>
<tr>
<td>Yoghurt (25)</td>
<td>2 (8)</td>
<td>EPEC</td>
<td>O128:H2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>STEC</td>
<td>O121:H7</td>
<td>-</td>
</tr>
</tbody>
</table>

*stx1*, shiga-toxin 1 gene; *stx2*, shiga-toxin 2 gene; *eaeA*, intimin gene; *hly*, hemolysin gene.
from raw milk or milk subjected to post-pasteurization contamination by food handlers mainly in developing countries with poor hygienic standards (WHO, 2015). Despite the negative result of salmonella species in all studies concerned with the microbiological examination of processed cheese worldwide (Kim et al., 2018), the culture method in our study revealed (Table 3) the presence of Salmonella in 3/25 (12%) of processed cheese samples that were serologically identified as S. Typhimurium, S. Infantis and S. Essen which globally reported as the most frequent cause of human salmonellosis and foodborne gastroenteritis outbreaks (Hendriksen et al., 2011). The presence of salmonella species in our result might be owned to the fact that processed cheeses encounter several conventional materials which have high potent to carry microbial contaminant of animal or human fecal wastes especially if it processed in unhygienic conditions at a lower temperature than that required for pasteurization (Kim et al., 2018).

It is worth noting that other investigated dairy products in Mansoura city were in harmony with our finding, where S. Typhimurium and S. Infantis were observed in 4% of soft cheese and Kareish cheese by El-Baz et al. (2017) and recently three isolates of S. Typhimurium were isolated from Kareish cheese by Elalify et al. (2019). To author knowledge, this is the first study reporting the isolation of very rare S. Essen serotype from the milk-based product as it commonly isolated only from retail food and chicken meat (Menghistu et al., 2011; Wang et al., 2015) and was of public health importance for both human and veterinary medicine.

Of note, the pathogenicity potential of Salmonella spp. depends mainly on the presence of the genetic determinants responsible for their virulence (Rhen et al., 2007; Sains et al., 2019). In the current study, PCR based assay proves the ability of isolated S. Typhimurium and S. Infantis to invade, destroy, escape from macrophage and colonize host intestinal cell inducing various lesions and gastrointestinal signs if consumed within infected dairy products as they were positive for invA, stn and spvC virulence genes (Table 3, Figure 2) which were also identified previously by El-Baz et al. (2017), Omar et al. (2018) and Elalify et al. (2019) in the same serotypes but in other conventional milk products. Astonishingly, PCR screening in our work confirmed that S. Essen serotype poses infection risks to humans as despite being negative for spvC virulence gene which responsible for systemic infection, it was positive for invA and stn virulence genes which are mandatory for Salmonella enterotoxigenic potency in inducing humans gastroenteritis.

Table 3. Prevalence, Serodiagnosis, and Virulence genes of Salmonella spp. isolated from processed cheese that consumed in Mansoura university hostels and hospitals.

<table>
<thead>
<tr>
<th>Type of samples (No.)</th>
<th>Prevalence No. (%)</th>
<th>Pathotype</th>
<th>Group</th>
<th>Somatic (O) antigen</th>
<th>Flagellar (H) antigen</th>
<th>Virulence genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Processed cheese (25)</td>
<td>S. Typhimurium B</td>
<td>1,4,5,12</td>
<td></td>
<td>I</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>S. Infantis C</td>
<td>6,7</td>
<td></td>
<td>R</td>
<td>1,5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>S. Essen B</td>
<td>4,12</td>
<td>g,m</td>
<td>g,m</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

invA, invasion protein gene; stn, Salmonella enterotoxin gene; spvC, Salmonella plasmin virulence gene.

Conclusions

Conclusively, This study declares the existence of highly pathogenic non-O157 VTEC and Salmonella spp. in dairy products every day supplied to the resident in Mansoura hospitals and hostels which constitute a potential risk to the public health. Hence, the implementation of good hygiene together with hazard analysis, and risk-based preventive control measures are rigorously required in the process of HACCP plan to eliminate the risk of contamination during processing rather than reliance on end-product testing.

References


Notes 7: 217.
Microbiol 41:1827-32.